The influence of testosterone and estrogenic compounds on microbial activity

An Honors Thesis (BIO 498)

By

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Abstract

Estradiol, estrone, and testosterone are pharmaceutical compounds that are commonly excreted and released into waterways where their fate is largely unknown. This study quantified the effect of these three hormone compounds on microbial activity in freshwater sediment. Sediment samples were collected from the White River in Muncie Indiana and exposed to varying concentrations of one of the three target hormones. Microbial activity was quantified as total respiration and nitrogen and phosphate uptake rates per unit dry mass. Increasing estrone concentration significantly decreased both respiration and nitrate uptake when compared to controls. Estradiol did not influence microbial respiration, but decreased nitrate uptake rates. Conversely, testosterone did not influence microbial respiration or nutrient uptake rates. Phosphate uptake was not significantly influenced by any experimental concentrations of the hormones tested. These results indicate that estrogenic compounds vary in their potential environmental effects. Further, hormones had pronounced effects on microbial activity even at trace concentrations currently measured in natural ecosystems. Thus, current estrogenic compound concentrations could be adversely affecting freshwater integrity.

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Introduction

I. Use of Human Sex Hormones

Human sex hormones, including estrogenic compounds (primarily estradiol or estrone) and testosterone are widely used for medical treatments including oral contraception (Mayo Clinic Staff Jan 2008), menopausal symptoms (Mayo Clinic Staff Feb 2008), transgender hormone replacement therapy (Asscheman and Gooren 1992), and sexual dysfunction (Davis 2008). 11.6 million women in the United States used oral contraceptives in the year 2002, and 44.5 million U.S. women, about 82%, have used oral contraceptives at some point during their

lives (NSFG 2002). Hormone replacement therapy used to treat the symptoms of menopause is still being widely researched and conflicting results about the long-term effects of HRT has caused concern; yet, there are an estimated 57 million prescriptions for menopausal hormone replacements filled each year in the United States (Fox 2008). It is also estimated that at least 1:2500 individuals in the United States is undergoing transgender hormone replacement therapy (Conway 2002). Of the adult males over 50 years old in the U.S., ~20% are affected by testosterone deficiency, nearly 8% of which are receiving testosterone replacement therapy (Carruthers 2009).

Oral contraception is the use of female sex hormones to prevent egg release. This type of birth control also thickens the cervical mucous, preventing easy passage of sperm or egg into the uterus. Thus, if taken as directed, oral contraception is effective at preventing pregnancy (Mayo Clinic Staff 2008). Estrogen treatments are also used as hormone replacement therapy associated with menopause (Mayo Clinic Staff 2008). Sex hormones, both estrogenic and testosterone are also used as hormone replacement therapy for transgender patients. Treatment with hormones yields development of sex characteristics specific to the desired gender. For example, breast formation is stimulated by estrogen treatment, while increased muscle mass may be brought on by taking testosterone treatments (Asscheman and Gooren 1992). Further, both testosterone and estrogenic compounds result in redistribution consistent with each associated gender (Asscheman and Gooren 1992).

Finally, testosterone can be used to help people who experience some forms of sexual dysfunction. Testosterone is linked to many aspects of sexuality (particularly in males, but not exclusively) and a low level of testosterone can affect sex drive; thus, men and women with low sex drive are often treated with testosterone. Also, erectile dysfunction and infertility can be linked to low testosterone levels in men (Davis 2008). Besides the legal medicinal uses for testosterone, many athletes utilize synthetic forms of testosterone, called anabolic-androgenic steroids, to promote muscle growth and improve athletic performance (Kishner and Svec 2008). While some legitimate medical uses exist for these types of drugs, many people may abuse them; thus an accurate estimate of use is unknown.

II. Fate of Hormones in the Environment

As with any pharmaceutical, humans metabolize only a fraction of the drug ingested with the remainder being excreted into wastewater (Koplin et al. 2002). Thus, with this widespread use of hormones, humans are excreting large amounts of these hormones into wastewater. In most municipalities, wastewater is routed to natural water bodies after treatment; or in the case of overflow during high flow events, prior to treatment (Radjenovik et al. 2007). Wastewater treatment plants are designed to remove primarily bacterial pathogens and nitrogenous waste (Radjenovik et al. 2007) and little information is available regarding the effectiveness of treatment in removing trace organic compounds like hormones and other pharmaceuticals. Because treatment plants are not designed to remove these chemicals, these compounds likely enter freshwater ecosystems after treatment. Pharmaceutical hormones in the environment are a concern because they may negatively influence freshwater integrity and they may enter into drinking water systems resulting in unintended human exposure.

According to a study assessing streams across the United States, estradiol is detectable in several forms. The least common form of estradiol (α-estradiol) was found at trace concentrations ~0.005 μg/L; the most common form (β-estradiol) was found at concentrations two orders of magnitude higher (~0.5 μg /L). Estrone and testosterone were also detected at trace concentrations of 0.005 μg /L (Koplin et al. 2002). Another study assessing waters in and around Hawaii calculated estrone concentrations in raw sewage to be as high as ~0.077 μg /L and ~0.0005 μg /L in receiving freshwaters (Atkinson et al. 2003). As one would expect, water samples collected in open waters, such as oceans, tended to have lower concentrations of estrone than inland waters (Table 1). This study also looked at bodies of water in other areas of the world, including Florida. Depending on the proximity to possible sewage pollution sites, Florida waters had estrone concentrations ranging from ~5.2×10^{-5} μg /L in open water areas to ~0.0016 μg /L in lagoons and bay areas (Atkinson et al. 2003). A study of the Thames River in Ontario, Canada also documented measurable concentrations of hormones. Estradiol was found at concentrations of 0.006-0.014 μg /L, and estrone was found at concentrations of 0.016-0.049 μg /L (Lishmana et al. 2006). These studies document the ubiquity of testosterone and estrogenic compounds in freshwater, due most likely to wastewater overflow pollution by municipal water treatment facilities. Because pharmaceuticals like sex hormones are designed to have a physiological effect on humans, it is likely that these compounds may also influence other organisms.

III. Potential effects of residual hormones on organisms

In humans, hormone activity in fertile young women is linked to autoimmune diseases (Cutolo et al 2004). Increased estrogen may activate a complex pathway in the immune system that increases the production of leukocytes in tissue and contributes to autoimmunity. Thus, patients with Rheumatoid arthritis and lupus often have elevated levels of estrogen in their blood.

Female reproductive cycles are also linked to the occurrence of respiratory distress in asthmatic patients (Riffo-Vasquez et al. 2007). Women admitted to the hospital for worsening asthma problems were often either menstruating or were within days of starting their menses. Thus, there is a correlation between rising estrogen and progesterone levels and worsening asthma symptoms. To test this correlation, mice were sensitized to ovalbumin, a protein substance that induces airway sensitivity, and then some had their ovaries removed (while others had theirs sham-removed). Mice that had their ovaries removed before or after sensitization to ovalbumin showed reduced immune responses compared to mice whose ovaries were left intact, indicating that the hormones produced by the ovaries aided in triggering an immune response.

Other vertebrates have also been influenced by hormone exposure. Specifically, rats treated with estrogenic compounds are less likely to have kidney stones than when treated with testosterone (Iguchi et al 1999). This relationship may also account for why kidney stones are more common in men than in women.

A study testing the effects of estrogenic hormone compounds on fish found that exposure to even small amounts of estrogenic compounds could result in feminization of the fish as well as possible immunologic and genetic malfunctions (Liney et al. 2006). Some exposed fish were unaffected, and developed normally as males, while others developed sexually undifferentiated, and still others developed as males with some female sexual organs. Also, fish exposed to the estrogenic compounds tended to grow larger than their control counterparts. Exposure to these compounds could also cause varying degrees of immunosuppression and damage to single stranded DNA.

Studies assessing the influence of acute exposure to pharmaceutical compounds at trace environmental concentrations are just beginning and potential long-term effects of chronic exposure to hormones in the natural environment remain unknown. What is known is that microbes are an important part of the hormones journey through the ecosystem (Ke et al. 2007). Bacteria are important for initially breaking down compounds like hormones and making products that are relatively inert. For example, bacteria in a tropical aquifer were found to degrade common estrogenic compounds but variability in degradation occurred among natural and synthetic compounds (Ke et al. 2007). *Novosphingobium tardaugens*, a bacterium found in activated sludge, was the first bacterium discovered that could break down estrogen in the form of estriol (Ke et al. 2007). *Fusarium proliferatum,* a soil-dwelling fungus (De Hoog, G.S. 2000), was then discovered to degrade synthetic forms of estrogen (ethynylestradiol) in natural ecosystems (Ke et al. 2007). Later still, *Rhodococcus zopfii* and *Rhodococcus equi*, bacteria common in soils and in herbivorous digestive tracts (Rabaud et al. 1995), were isolated and found to degrade all forms of estrogen detected in the aquatic environments (Ke et al. 2007). Though the study was done in a marine environment, evidence exists that some estrogen-degrading bacteria thrive in many other environments (Ke et al. 2007). Without these bacterial interactions with estrogen compounds, the hormones would remain unchanged in the environment and persist for longer periods of time.

While some microorganisms can degrade hormone compounds, it is generally observed under controlled conditions. Therefore, observation of how natural microbial communities react to varying concentrations of hormones remains to be evaluated. The objective of this study was to quantify how increasing the concentrations of different hormones will affect the activity of naturally occurring freshwater sediment microbial communities

We hypothesized that higher concentrations of estradiol, estrone, and testosterone would increase microbial activity. Increasing hormone concentration will increase the amount of degradable substrate usable by microbes and potentially resulting in higher rates of respiration.

Methods

I. Microbial Respiration

Microbial respiration is the route by which organisms break down carbon-based molecules to obtain energy for life processes (Phelan 2009). Cellular respiration occurs in three steps: glycolysis, the Krebs cycle, and the electron transport chain. As the carbon source, generally glucose, passes through the three steps of cellular respiration, carbon-carbon bonds are broken and the resulting energy is harnessed in adenosine triphosphate (Phelan 2009). Oxygen is a necessary element of cellular respiration because it acts as a final electron donor during the electron transport chain and produces water molecules (Phelan 2009). Thus, oxygen consumption can be used to evaluate the amount of cellular respiration that has occurred during a reaction.

II. Collection of Sediment

Sediment and water were obtained from a chosen site along the White River in Muncie, IN for measurement of microbial respirations. The White River was an ideal choice of waterway because it is the waterway used for deposition of sewage overflow from the city of Muncie through combined sewer overflows. The deposition of sewage into the river makes it a likely source of water affected by pharmaceutical compounds. Additionally, the White River is a drinking water source for central Indiana communities. Sediment was collected from the shallow shoreline area of the river (~5 cm top sediment), avoiding rocks, leaves and branches. After collection, sediment was returned to the laboratory and homogenized through a USGS no. 5 sieve before being distributed into individual labeled falcon tubes for microbial activity assays.

III. DHA Analysis

2 mL of homogenized sediment and 2.5 mL of river water were placed into each of 78 falcon tubes (15 mL). Five replicates each of five treatment concentrations each for estradiol, estrone, and testosterone were prepared in addition to control samples with no hormone addition. The tubes were refrigerated overnight and subsequently vortexed. After overnight equilibration, hormone treatments were added to obtain the effective target concentrations (Table 2.1). To all 78 falcon tubes, 1 mL of 0.75% Iodonitroetrazolium (INT) Chloride was added to act as the final electron acceptor for microbial respiration in conjunction with dehydrogenase enzyme activity assays (Smith and McFeters 1997, Hill et al. 2002). The tubes were capped and vortexed, then incubated at room temperature for 3 h.

After the 3 h incubation, 8 mL of methanol was added to each tube to halt the reaction and each tube was capped and vortexed. Each tube was then centrifuged for 5 min and the supernatant was pipette into cuvettes and absorbance measured using a UV spectrophotometer at 428 nm. The remaining supernatant was drained and the samples were air dried for 4 d before weighing to obtain a sediment dry weight. Several empty falcon tubes were weighed and averaged to give an average tube mass to subtract from each dry weight, giving the grams dry mass for each replicate. A standard curve was established for calculation of the consumption of oxygen by adding INT formazan to methanol at variable concentrations (Table 2.2). The standard curve samples were also measured for absorbance using a UV spectrophotometer at 428 nm.

IV. Nutrient Assimilation

6 mL of homogenized sediment and 6 mL of unfiltered river water were placed into each of 78 falcon tubes as for dehydrogenase activity assays. Five replicates of 5 concentrations each for estradiol, estrone, and testosterone were tested with 3 control replicates (no hormone addition). For nutrient assimilation assays, 5 initial water samples were filtered and frozen for subsequent measurement of nutrient concentrations. The samples were incubated for 4 d at room temperature. Following incubation, the samples were vortexed, then centrifuged. The supernatant was removed and filtered into tubes for analysis of nutrient concentrations. All filtered water samples were analyzed for nutrient concentrations (nitrate and phosphate) using a DIONEX Ion Chromatograph. Nutrient uptake was calculated as the change in nutrient concentrations (initial concentration-final concentration) for each replicate expressed per mass of sediment and time.

V. Statistical Analyses

Analysis of Variance (ANOVA) was conducted to identify significant differences in microbial activity among treatments with hormone concentrations as the main effect. Additionally, linear and non-linear dose response curves were developed to assess microbial response patterns with increasing hormone concentrations. All statistical analyses were conducted using MiniTab statistical software.

Results

Increases in estradiol and testosterone concentrations did not significantly affect microbial respiration in White River sediment ($P>0.1$; Figure 1A,C). In contrast, an increase in estrone concentration caused a significant decline in microbial respiration ($P<0.001$; Figure 1B). Respiration with estradiol exposure ranged from 4.1 to 7.3 moles of oxygen consumed; respiration with testosterone exposure ranged from 4.1 to 6.7 moles of oxygen consumed; and, respiration with estrone exposure ranged from 3.3 to 6.7 moles of oxygen consumed. Phosphate uptake was not significantly affected by the addition of hormones at any concentration tested ($P>0.10$; *data not shown*). However, nitrate uptake was decreased with increasing concentrations of both estradiol and estrone ($P = 0.0019$; Figure 2A and $P = 0.0008$; Figure 2B , respectively). Increasing testosterone concentration did not significantly influence nitrate uptake ($P>0.10$; Figure 2C).

Discussion

Overall, trace-concentrations of estrogenic compounds were found to influence river sediment microbial communities. However, microbial response varied both with compound tested and measure of microbial activity. Estrone had the most pronounced effects on microbial activity affecting both respiration (Figure 1B) and nitrate uptake (Figure 2B). In contrast, trace-concentrations of estradiol only influenced microbial nitrate uptake (Figure 2A). For all experiments and treatments, microbial activity (as respiration and nutrient uptake) was measurable and thus compounds did not completely inhibit activity. Thus, these compounds may be degradable by microbes in the sediment at trace environmental concentrations. Effective toxic concentrations were not reached at the hormone concentrations tested, though oxygen consumption declined slightly at the highest concentration tested for estradiol (1.0 μg/L; Figure 1A). Estrone, however, likely has a lower effective toxicity concentration, as decreases in microbial respiration were apparent even at the lowest treatment concentrations (Figure 1B). Likewise, estrone exposure yielded decreased nitrate uptake even at the lowest concentrations tested (Figure 2B).

The decrease in microbial activity with addition of increasing estrone concentrations indicates that estrone inhibits microbial activity. However, estradiol and testosterone did not

influence respiration. Respiration is a holistic measure of microbial activity. Thus, changes in respiration may result from changes associated with all prokaryotes or from a specific group (e.g., nitrifiers, decomposers). It is unclear what mechanism may have yielded decreased respiration associated with trace-concentrations of hormones in this study. There are few studies that have focused on microbes as the target organism, potentially limiting a comprehensive understanding of microbial interaction with hormones. Also, relatively few studies have been done to evaluate the toxicity of estrone (U.S. EPA 2010) compared to the number of studies for estradiol (U.S. EPA 2010), possibly resulting in an incomplete understanding of the compounds.

At lower estradiol concentrations, nitrate uptake increased, but when the concentrations approached ~0.5 μg/L, nitrate uptake decreased (Figure 2A). Likewise, increasing estrone concentration resulted in an increase in nitrate uptake at low concentrations, but at a concentration of ~0.3 μg/L, nitrate uptake decreased rapidly (Figure 2). These non-linear trends are relevant because they demonstrate that hormone exposure can result in complex non-linear responses and not strictly simple linear declines in microbial activity. At low concentrations, hormones do not impede nitrate uptake, in fact they stimulate nitrate uptake, but after concentrations reach a certain threshold, microbial activity begins to decline.

These data indicate that if estrone and estradiol concentrations increase in natural waterways, the ecosystem could be influenced by alterations in microbial activity. Also, if hormone levels are affecting microbes, it is likely that other organisms may also be influenced, potentially causing further changes in the ecosystem. As pointed out by Koplin et al. (2002) and Radjenovik et al. (2007), wastewater treatment facilities are not designed to remove pharmaceutical compounds. Thus, further measures may be needed to mitigate wastewater contamination of trace pharmaceuticals and further research should be done to evaluate which specific compounds may pose a threat to ecosystems.

These data may act as a prediction of future effects if hormone concentrations in natural waterways continue to increase. Previous studies have quantified hormone concentrations in natural waterways as high as ~0.05 μg/L, consistent with low treatment concentrations used in this study. If organisms in the environment do not degrade hormone compounds, then these compounds could continue to accumulate and increase in concentration threatening freshwater

integrity and potentially human health through unintentional consumption associated with drinking water.

Literature Cited

Asscheman, H. Gooren L.J.G. 1992. Hormone treatment in transsexuals. TransGenderCare. 6 Sept 2009. http://www.tgcare.com/medical/hormonal/hormone-tx_assch_gooren.htm.

Carruthers, M. 2009. Time for international action on treating testosterone deficienty syndrome. *The Aging Male*. 12(1): 21-28.

Conway, L. 2002. How frequently does transexualism occur?. 21 Sept 2009. http://ai.eecs.umich.edu/people/conway/TS/TSprevalence.html.

Cutolo, M. Capellino, A.S. Villaggio, B. Montagna, P. Seriolo, B. Staub, RH. 2004. Sex hormones influence on the immune system: basic and clinical aspects in autoimmunity. *Lupus*. 13: 635-638.

Davis, B. 2008. Testosterone. 29 May 2008. Healthwise Inc. 6 Sept 2009. http://men.webmd.com/testosterone-15738

De Hoog, C.S. Guarro, J. Gene, J. Figueras, M.J. Atlas of Clinical Fungi. ASM Press. 2nd ed.

Fox, M. 2008. Hormones raise breast cancer risk quickly. Thomas Reuters. 21 Sept 2009. http://uk.reuters.com/article/idUKN1440874820080115?pageNumber=1&virtualBrandChannel=0.

Hill BH, Herlihy AT, Kaufmann. 2002. Benthic microbial respiration in Appalachian Mountain, Piedmont, and Coastal Plains streams of the eastern USA. Freshwater Biology. 47, 185-194.

Iguchi, M. Takamura, C. Umekawa, T. Kurita, T. Kohri, K. 1999. Inhibitory effects of female sex hormones on urinary stone formation in rats. *Kidney International*. 56: 479-485.

Ke, J. Zhuang, W. Gin, K.Y. Reinhard, M. Hoon, L.T. Tay, J. 2007. Characterization of estrogen-degrading bacteria isolated from an artificial sandy aquifer with ultrafiltered secondary effluent as the medium. *Applied Microbiology and Biotechnology*. 75: 1163-1171.

Kischner, S. Svec, F. 2008. Anabolic steroid use and abuse. eMedicine. 21 Sept 2009. http://emedicine.medscape.com/article/128655-overview.

Koplin, D.W. Furlong, E.T, Meyer, M.T. Thurman, E. M. Zaugg, S.D. Barber, L.B. Buxton, H.T. 2002. Pharmaceuticals, hormones, and other organic wastewater contaminants in U.S. streams, 1999-2000: A national reconnaissance. *Environmental Science and Technology*. 36(6): 1202-1211.

Liney, K.E. Hagger, J.A. Tyler, C.R. Depledge, M.H. Galloway, T.S. Jobling, S. 2006. Health affects in fish of long-term exposure to effluents from wastewater treatment works. *Environmental Health Perspectives*. 114: 81-89.

Mayo Clinic Staff. 2008. The Pill and other hormonal contraceptives. Mayo Clinic. 6 Sept 2009. http://mayoclinic.com/health/birth-control/BI00014.

Mayo Clinic Staff. 2008. Hormone Therapy: Is it right for you. Mayo Clinic. 6 Sept 2009. http://www.mayoclinic.com/health/hormone-therapy/WO00046.

NSFG(National Surveys of Family Growth). 2002. Use of contraception and use of family planning services in the United States: 1982-2002.

Phelan, J. 2009. Cellular respiration. What is life? A guide to biology. W.H. Freeman & Co.

Rabaud, C. May, T. Hoen, B. Maignan, M. Gerard, A. Canton, P. 1995. Disseminated abscesses caused by Rhodococcus equi in a patient with chronic lymphocytic leukemia. Clinical Infectious Diseases. 20: 478-479.

Radjenovic, J. Petrovic, M. Barcelo, D. 2007. Analysis of pharmaceuticals in wastewater and removal using a membrane bioreactor. *Analytical and Bioanalytical Chemistry*. 387(4): 1365–1377.

Riffo-Vasquez, Y. Ligeiro de Oliveira, A.P. Page, C.P. Spina, D. Tavares-de-Lima, W. 2007. Role of sex hormones in allergic inflammation in mice. *Clinical and Experimental Allergy*. 37: 459-470.

Smith JJ and McFeters GA. 1997. Mechanisms of INT (2-(4 iodophyenyl)-3-(4-nitrophenyl)-5-phenyl tetrazolium chloride and CTC (5-cyano-2,3,-ditolyl tetrazolium chloride) reduction in E.coli k-12. Journal of Microbial Methods. 29, 161-175.

U.S. Environmental Protection Agency. 2010. Ecotox Database. U.S. Environmental Protection Agency. 20 April 2010. http://cfpub.epa.gov/ecotox/quick_query.htm

Yoshimoto, T. et al. 2004. Degradations of estrogens by Rhodococcus zopfii isolates from activated sludge in wastewater treatment plants. *Applied Environmental Microbiology*. 70(9): 5283-5289.

Tables and Figures

Table 1: Comparison of estrogenic compounds and testosterone concentrations in water sources. (Lishmana et al. 2006; Atkinson et al. 2003).

	Open Water	Lagoons/Bays	Streams/Rivers
Estradiol	n/a	n/a	0.005-0.05 μg /L
Estrone	$5.2x10^{-5}$ μg /L	0.0016 μg /L	0.005-0.049 μg /L
Testosterone	n/a	n/a	0.005 μg /L

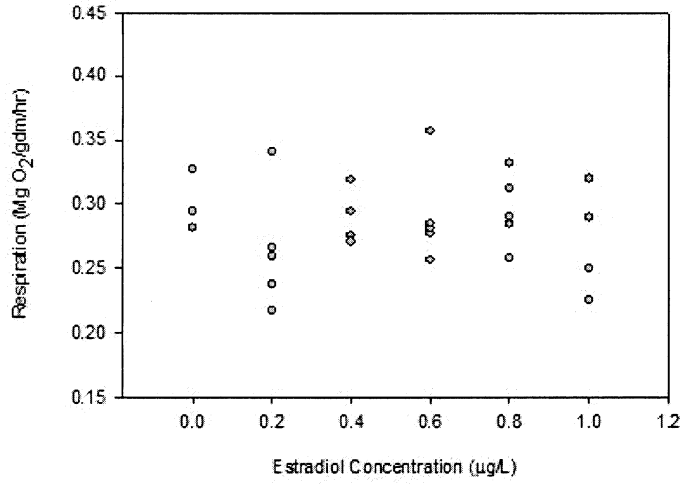
**n/a = data not available*

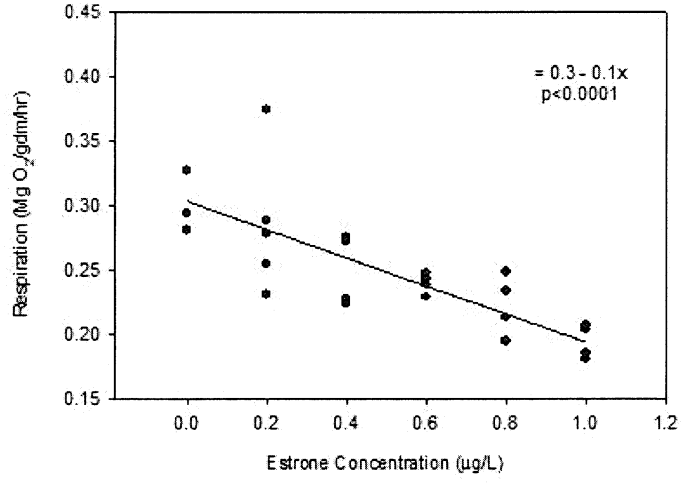
Table 2.1: Hormone treatments and volumes added to each appropriate replicate to achieve effective target concentrations.

Target Concentration	Estradiol (20 µg/L stock solution)	Estrone (10 µg/L stock solution)	Testosterone (50 µg/L stock solution)
0.2	45 µL	90 µL	18 µL
0.4	90 µL	180 µL	36 µL
0.6	135 µL	270 µL	54 µL
0.8	180 µL	360 µL	72 µL
1	225 µL	450 µL	90 µL

Table 2.2: Volumes of INT Formazan added for development of formazan standard curve, with corresponding absorbance values.

Concentration	mL of Methanol	mL of INT Formazan	Absorbance at 428nm
0	10	0	-0.089
2	10	0.6	0.304
4	10	1.2	0.573
6	10	1.8	0.779
8	10	2.4	0.952
10	10	3	1.104





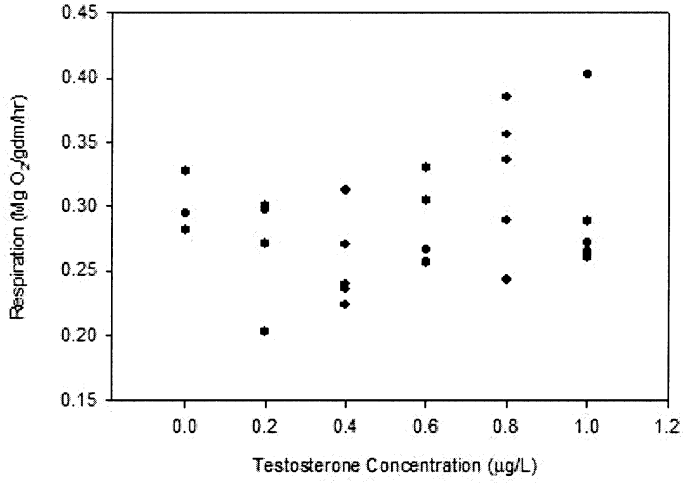
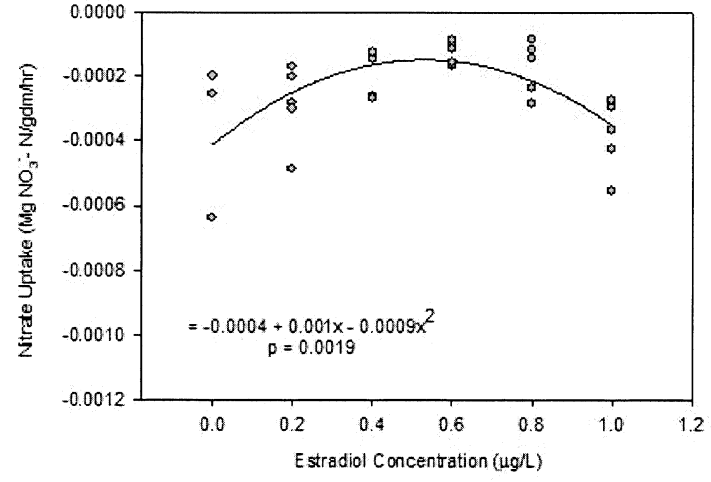
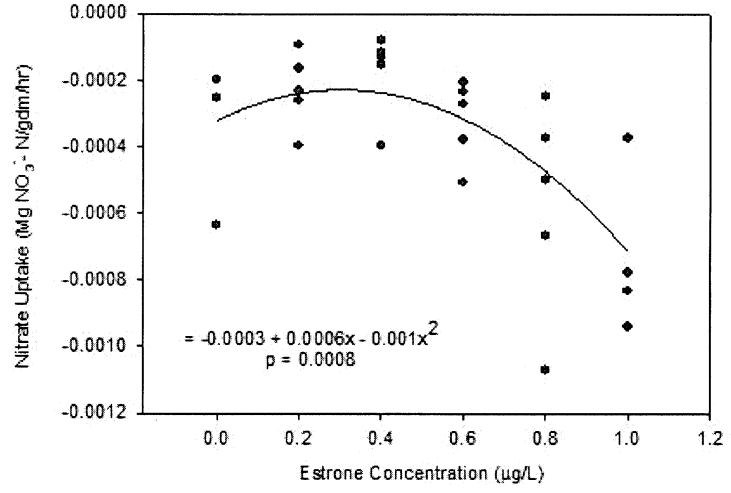


Figure 1: Effect of A) estradiol, B) estrone, and C) testosterone concentrations on microbial respiration.





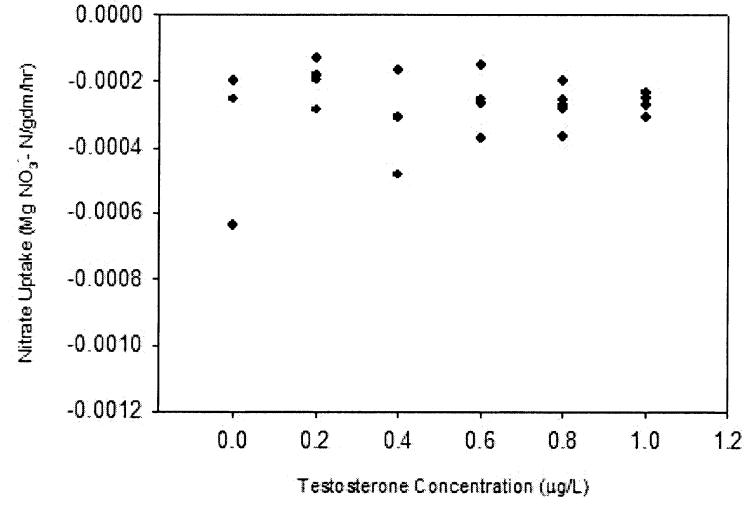


Figure 2: Effect of A) estradiol, B) estrone, and C) testosterone concentrations on nitrate uptake.